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Luteoloside attenuates neuroinflammation in focal cerebral ischemia in rats via regulation of the PPARγ/Nrf2/NF-κB signaling pathway

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ABSTRACT

Luteoloside, a flavonoid compound, has been reported to have anti-inflammatory, anti-oxidative, antibacterial, antiviral, anticancer, and cardioprotective effects, among others, but its neuroprotective effects have rarely been studied. The purpose of this study was to investigate the protective effect of luteoloside on cerebral ischemia and explore its potential mechanism. Middle cerebral artery occlusion (MCAO) was performed to investigate the effects of luteoloside on cerebral ischemia-reperfusion (I/R). Male Sprague-Dawley rats were randomly divided into six groups: sham, MCAO, luteoloside (20 mg/kg, 40 mg/kg, 80 mg/kg) and nimodipine (4 mg/kg). The results showed that luteoloside alleviated neurologic deficits and cerebral edema as well as improved cerebral infarction and histopathological changes in MCAO rats. Luteoloside significantly inhibited I/R-induced neuroinflammation, as demonstrated by reduced levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the brain tissues of MCAO rats. Furthermore, our results demonstrated that luteoloside significantly suppressed the activation of nuclear factor-kappa B (NF-κB) signaling, upregulated the protein expression of peroxisome proliferator activated receptor gamma (PPARγ) and increased NF-E2-related factor (Nrf2) nuclear accumulation in MCAO rats. Collectively, our findings suggested that luteoloside played a crucial neuroprotective role by inhibiting NF-κB signaling in focal cerebral ischemia in rats. Furthermore, PPARγ and Nrf2 were also important for the anti-inflammatory effect of luteoloside. In addition, our data suggested that luteoloside might be an effective treatment for cerebral ischemia and other neurological disorders.

1. Introduction

Ischemic stroke is one of the main causes of death and disability in developed countries [1]. Ischemic stroke occurs when blood supply to the brain becomes interrupted. Although cerebral ischemia causes high morbidity in the clinic, therapeutic options for acute ischemic stroke remain very limited, and few neuroprotective treatments have been successfully developed to prevent ischemic stroke.

Cerebral ischemia-reperfusion (I/R) injury after thrombolysis in stroke leads to the rapid death or apoptosis of neurons in ischemic penumbra. The process of neuronal injury after cerebral ischemia is very complicated, but an increasing number of studies has demonstrated that the inflammatory response plays an important role in the above process [2]. The increased production of inflammatory cytokines and proinflammatory mediators aggravates neurological injury and promotes apoptosis of neural cells following ischemia [3]. Therefore, preventing inflammation and apoptosis is a feasible strategy for ischemic stroke intervention.

Nuclear factor-kappa B (NF-κB) is a critical transcription factor that regulates inflammation and various autoimmune diseases [4]. Expression of a large number of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-lβ (IL-1β), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), can be regulated by the activation of NF-κB [5,6]. Studies suggest that NF-κB is activated in cerebral vascular endothelial cells, glial cells and neurons after cerebral ischemia, which triggers a dramatic increase in the expression of inflammatory cytokines, leading to an inflammatory cascade reaction and aggravating brain damage [7].

Peroxisome proliferator activated receptor gamma (PPARγ) is a ligand-activated nuclear transcription factor. PPARγ agonists have been reported to show anti-inflammatory and anti-oxidant effects in several models of central nervous system disorders, such as ischemic stroke,

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Alzheimer's disease and Parkinson's disease [8,9]. Several in vitro and in vivo studies demonstrate that PPARγ reduces proinflammatory cytokine release by inhibiting the nuclear transcription factor NF-κB [10,11].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that plays an important role in the response to oxidative stress. In recent years, increasing evidence has shown that activation of the Nrf2 pathway significantly inhibits NF-κB and plays a protective role in the inflammatory response [12,13]. In brief, the interaction of Nrf2 and the NF-κB pathway plays an important role in the development of cerebral ischemia pathology and has become an important neuropathologic mechanism and therapeutic target.

Luteoloside (also called cynaroside or luteolin-7-O-glucoside) is a flavonoid compound found in many plants and herbs. It has been reported that luteoloside has anti-oxidative, anti-inflammatory, antibacterial, antiviral, anticancer and other functions [14–17]. For example, recent studies showed that luteoloside exerted cardioprotective effects in vitro [18–20]. However, there are few reports on the neuroprotective effects of luteoloside.

Therefore, this study was designed to evaluate the protective effects of luteoloside on cerebral I/R injury induced by middle cerebral artery embolism (MCAO) and to investigate whether the inflammatory response mediated by PPARγ/Nrf2/NF-kB signaling is implicated in this process.

2. Materials and methods

2.1. Compounds and reagents

Luteoloside (purity > 98%) was obtained from Herbpurify Co., Ltd. (Chengdu, China). ToxinSensor™ Single Test Kit, GenScript Biotech Co., Ltd. (Nanjing, China). 2,3,5-Triphenyltetrazolium chloride (TTC) was obtained from Solarbio Science & Technology Co., Ltd., (Beijing, China). TNF-α and IL-1β Enzyme-Linked Immunosorbent Assay (ELISA) kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-IκBα, anti-p-IκBα, anti-p65, anti-p-p65, anti-PPARγ, anti-Nrf2 and lamin B antibodies were purchased from Cell Signaling Technology (Danvers, USA). Anti-β-action and anti-IgG were obtained from Proteintech Group, Inc. (Wuhan, China). Whole cell protein extraction kits were purchased from Solarbio Science & Technology Co (Beijing, China), and nuclear and cytoplasmic protein extraction kits were purchased from Beyotime Biotech Inc., (Nantong, China). Other reagents were analytically pure.

2.2. Animals

Male rats (250–270 g) were purchased from the Center of Experimental Animals in Henan province (Zhengzhou, China). The rats were kept in a controlled environment (temperature: 25 ± 2 °C; humidity: 60 ± 5%, 12 h dark/light cycle). The animals had free access to food and water. All studies were carried out in compliance with the ethical committee for Laboratory Animals Care and Use of Henan University.

2.3. Establishment of the animal model of cerebral ischemia

The animals were deprived of food for 12 h before the MCAO procedure was performed. The rats were anesthetized with an intraperitoneal injection of chloral hydrate (350 mg/kg). MCAO was performed as previously described [21–23]. Briefly, a nylon monofilament suture was inserted 18 mm into the left internal carotid artery. Reperfusion was allowed after 2 h by withdrawal of the monofilament. In sham-operated animals, the middle cerebral artery was not occluded.

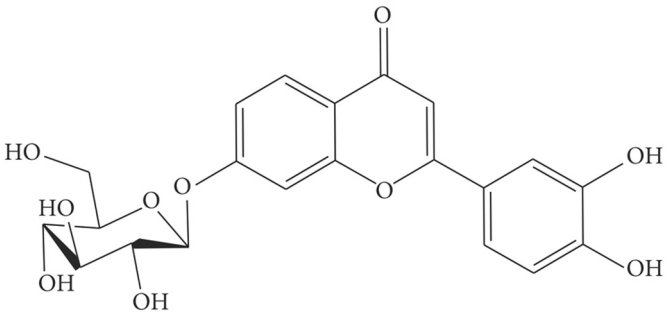

Fig. 1. Chemical structures of luteoloside.

2.4. Drug preparation and administration

The chemical structure of luteoloside is shown in Fig. 1. The Limulus Amebocyte Lysate (LAL) Gel Clot Assay was used to evaluate luteoloside samples for possible endotoxin contamination. The assay sensitivity of 0.03 EU/ml was confirmed for all kits used. The test procedure was performed following the manufacturer's instructions. The results showed that the content of endotoxin in luteoloside was lower than 0.06 EU/mg, which was in accordance with the regulations. Luteoloside was dissolved in DMSO; the final concentration of DMSO was < 1%. Designated doses of luteoloside and nimodipine were intraperitoneally injected immediately and 12 h after MCAO surgery. The sham and MCAO group rats were treated with an equal amount of 0.9% normal saline. After reperfusion for 24 h, the animals were sacrificed, and the infarct volume in the brain was determined by TTC staining. Alternatively, ischemic penumbra containing the cortex and hippocampus were separated and then lysed for inflammatory cytokine and Western blot analysis.

2.5. Neurological deficit assessment

A modified neurological severity score test was performed to assess the effects of luteoloside on neurological dysfunction in rats after MCAO surgery. The neurological score was evaluated 24 h after reperfusion by an observer blinded to the animal groups as described previously [24]. The neurologic score was assigned as follows: 0, no deficit; 1, flexion of contralateral forelimb; 2, decrease in resistance toward the contralateral plane; 3, circling monolaterally; and 4, labored or absent ambulation. Higher scores represented more serious dysfunction.

2.6. Evaluating cerebral edema

Cerebral edema was detected according to previously published methods [25]. The wet weight of brain samples was immediately measured on an electronic analytical balance. Then, the tissues were placed in an incubator at 100 °C for 48 h to obtain the dry weight. Cerebral edema = (wet weight − dry weight) / wet weight × 100%.

2.7. Histological examination

After reperfusion for 24 h and after the behavior tests, the rats were anesthetized with 10% chloral hydrate and then perfused through the left ventricle with 4% paraformaldehyde. The brains were removed and immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h at 4 °C. Brain blocks were embedded in paraffin and cut into 5 μm coronal sections. HE staining was performed using a Hematoxylin and Eosin Staining Kit (Beyotime, China), according to the manufacturer's instructions.

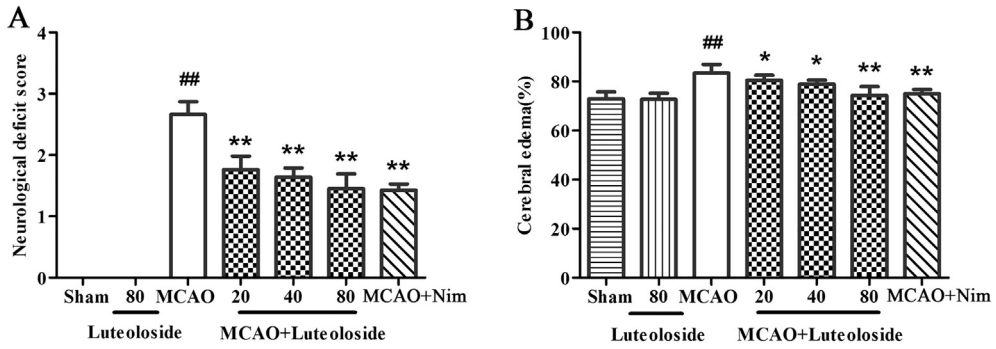

Fig. 2. The effects of luteoloside on neurological deficit scores and cerebral edema in MCAO-treated rats. A: the neurological deficit score; B: cerebral edema. Data are represented as the mean ± SD, n = 8, $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus MCAO group.

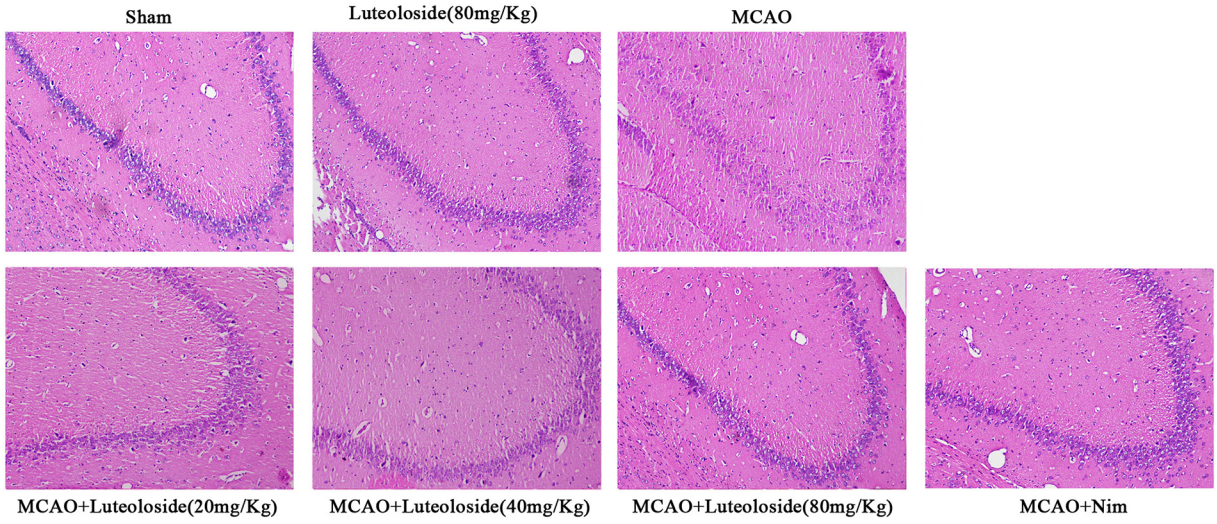

Fig. 3. The effects of luteoloside on histopathological changes in the brain of MCAO-treated rats determined by HE staining (magnification, ×100). Representative images are chosen from each experimental group.

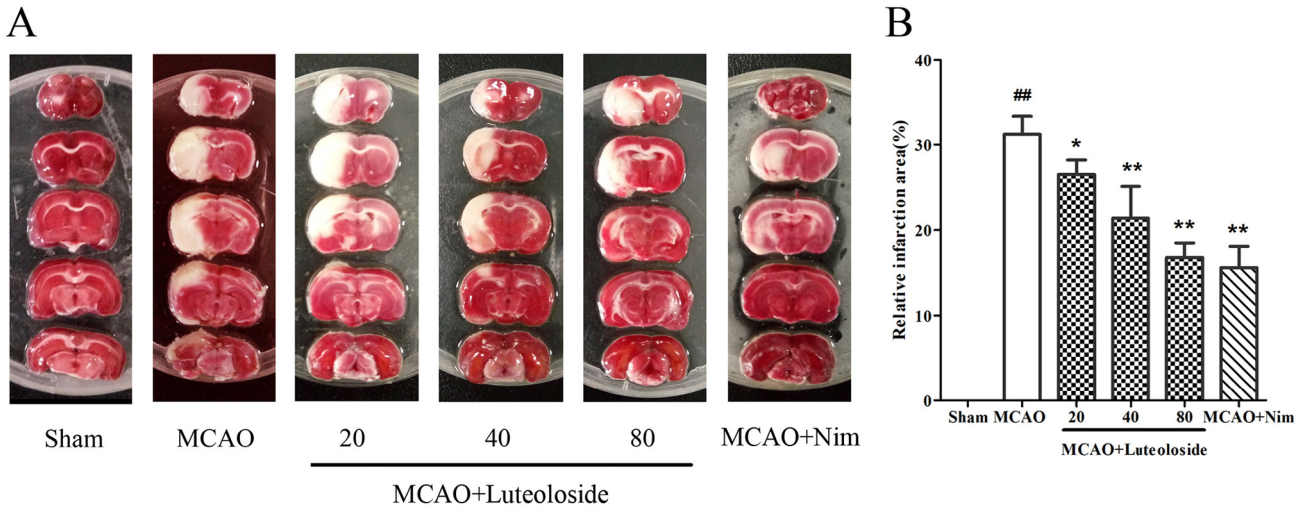

Fig. 4. The effects of luteoloside on cerebral infarction in MCAO-treated rats. After reperfusion for 24 h, the animals were sacrificed, and infarct volume in the brain was determined by TTC staining. A: Typical images were chosen from each experimental group. B: Quantitative analysis of the infarcted brain regions. Data are represented as the mean ± SD, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus MCAO group.

2.8. Evaluating the rat brain infarct area

After reperfusion for 24 h, all animals were sacrificed. Brain infarct areas were quantified utilizing the TTC staining technique. The brains were gently removed and frozen at −20 °C for 10 min and then sliced into 2-mm thick coronal sections. The slices were incubated in a 2% solution of TTC in saline phosphate at 37 °C for 30 min; then, the brain slices were arranged in order. Digital cameras were used to capture the positive and negative parts of the brain; the red-colored regions represent normal tissue, while the unstained regions represent infarct

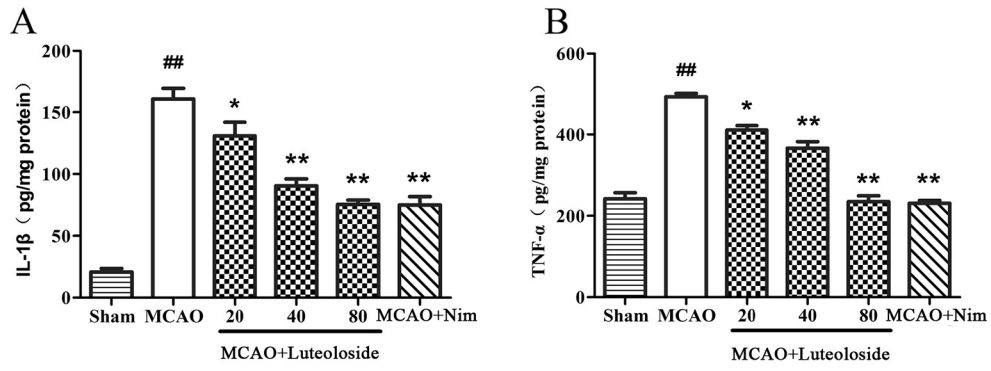

Fig. 5. Effect of luteoloside on the release of proinflammatory cytokines in the MCAO rat brain. The levels of IL-1β (A) and TNF-α (B) were detected by ELISA. The expression of iNOS (C) and COX-2(D) were measured by Western blot. Protein expression was normalized to that of β-actin, and relative densities were normalized to the sham group. The values presented are the means ± SD of three independent experiments. $^{\#\#}P < 0.01$ compared with sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with MCAO group.

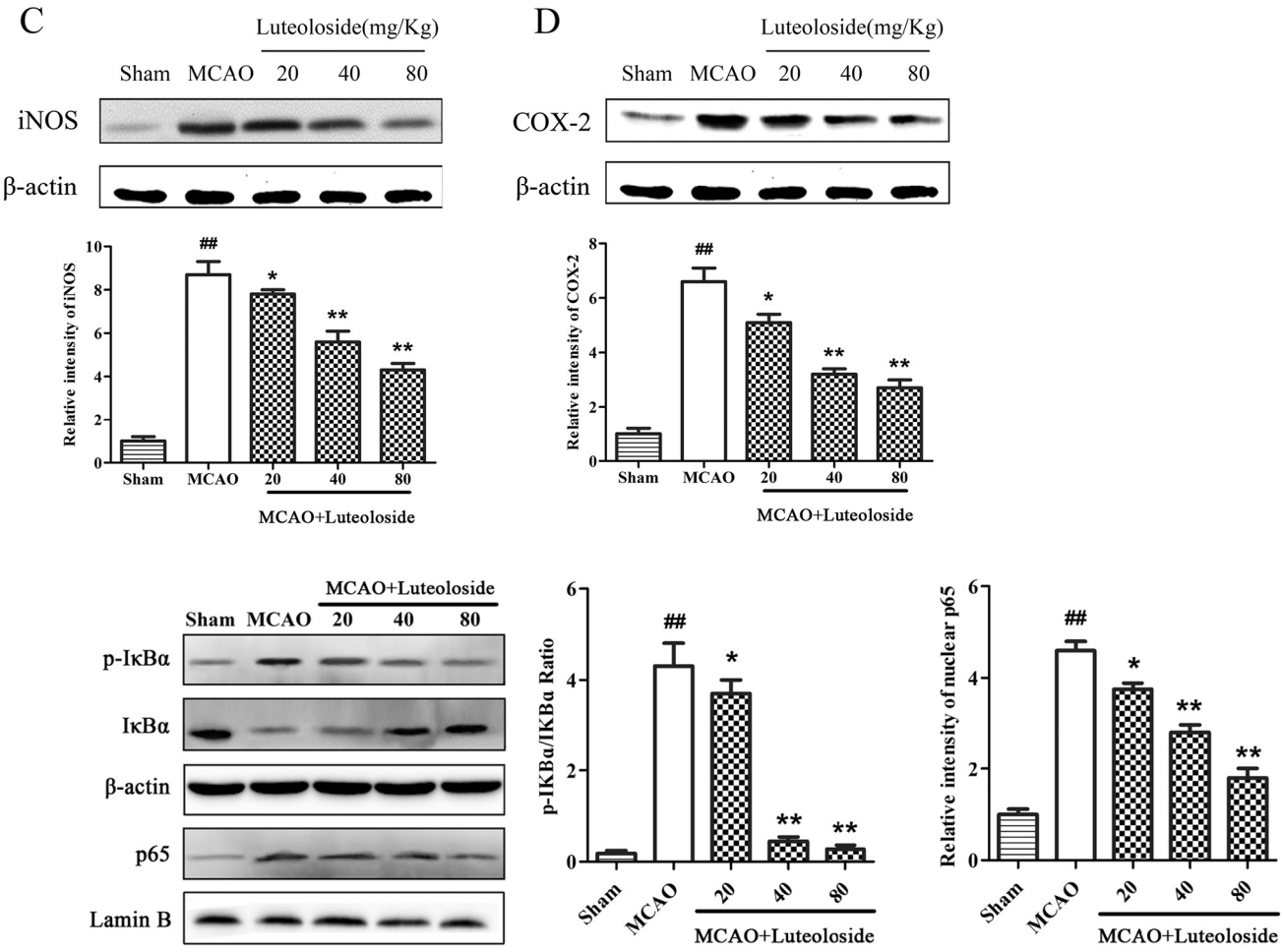

Fig. 6. Effect of luteoloside on the MCAO-induced NF-κB activation. The expression of IκBα and NF-κB p65 were detected by Western blot using specific antibodies in rat brains. The values presented are the means ± SD of three independent experiments. $^{\#\#}P < 0.01$ compared with sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with MCAO group.

areas. The volume of the whole brain and the infarct tissue was determined with Image-Pro Plus software (Media Cybernetics, USA). The following formula was used to calculate the percent infarct: (total area of contralateral hemisphere) − (area of uninfarcted area of ipsilateral hemisphere) / total area of contralateral hemisphere × 100%

2.9. Detection of inflammatory cytokines in brain tissue

The levels of TNF-α and IL-1β in brain tissue were measured using ELISA kits according to the manufacturer's instructions. Flash-frozen brains from treated rats were homogenized and lysed to perform ELISA from tissue lysates. Then, the absorbance of each well was read at 450 nm with a microplate spectrophotometer (Thermo, Multiskan Ascent, USA).

2.10. Western blot analysis

After MCAO surgery, the rat brain tissues were subjected to protein expression analysis. Nuclear and cytoplasmic protein extraction kits were used to isolate cytosolic and nuclear protein fractions. The brain tissues were homogenized, washed with PBS, and incubated in lysis buffer. The proteins were collected from the supernatant after centrifugation at 12,000 × *g* for 10 min, and their concentrations were determined using a BCA protein assay kit. The samples were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk and

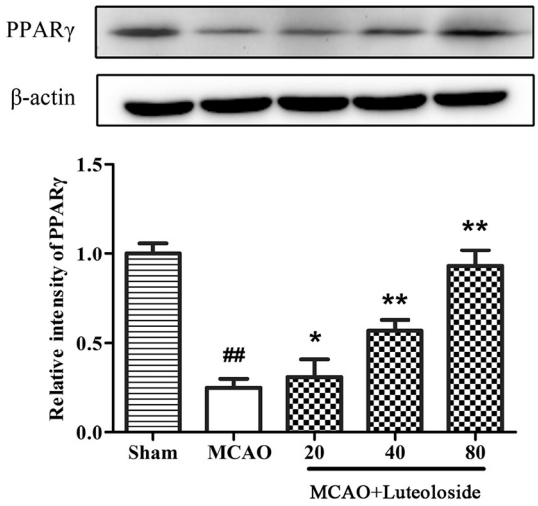

Fig. 7. Effect of luteoloside on the PPARγ expression in the MCAO rat brain. Protein expression was normalized to that of β-actin, and relative densities were normalized to the sham group. The values presented are the means ± SD of three independent experiments. $^{\#\#}P < 0.01$ compared with sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with MCAO group.

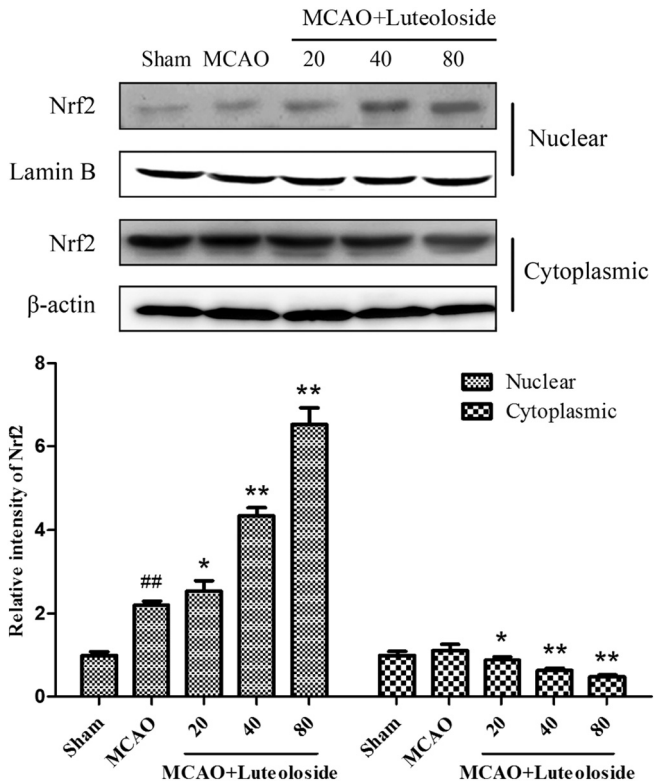

Fig. 8. Effects of luteoloside on the Nrf2 expression in both cytoplasm and nucleus in the MCAO rat brain were measured by Western blot. Protein expression was normalized to that of β-actin or Lamin B, and relative densities were normalized to the sham group. The values presented are the means ± SD of three independent experiments. $^{\#\#}P < 0.01$ compared with sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with MCAO group.

then incubated at 4 °C overnight with primary antibodies that recognize iNOS, COX-2, IκBα, p-IκBα, NF-κBp65, PPARγ, Nrf2, β-actin, or lamin B. After washing with tris-buffered saline-tween 20 (TBST), the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1.5 h at room temperature. Finally, the membranes were washed with PBS-T and then developed with an enhanced chemiluminescence (ECL)-plus kit, and they were detected using ImageJ2x software (National Institutes of Health, Bethesda, MD). Three samples were tested and representative results were shown.

2.11. Statistical analysis

All data were presented as the mean ± SD. Comparison between groups was assessed by one-way ANOVA, followed by Dunnett's post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Luteoloside improved neural function and decreased damage and cerebral edema induced by MCAO in the rat brain

First, the effects of luteoloside on neural function in rats were evaluated by neurologic scores. There was no difference in the scores of the rats in the luteoloside group compared with those in the sham group ($P > 0.05$), and the scores of the MCAO rats were significantly increased ($P < 0.01$). However, treatment with either luteoloside or nimodipine significantly decreased the scores compared with the MCAO-treatment group (Fig. 2A).

Next, the results showed that the administration of luteoloside to normal rats did not affect brain water content. However, the ratio of cerebral edema was significantly increased in the MCAO group compared with that in the sham group. In contrast, the increased cerebral edema in MCAO rats was markedly suppressed by treatment with either luteoloside or nimodipine (Fig. 2B).

3.2. Luteoloside improved the histopathological changes in MCAO-treated rat brains

Compared with rats in the sham group, the rats that only received luteoloside exhibited normal brain histological architecture. However, HE staining showed that the number of nerve cells in the MCAO group was significantly reduced, the cells appeared smaller and disordered, and the intercellular space was dilated compared with the sham group. Luteoloside significantly improved these morphological changes induced by MCAO (Fig. 3).

3.3. Luteoloside alleviated MCAO-induced cerebral infarction

After 24 h of MCAO surgery and after the behavioral test, the animals were sacrificed, and the extent of ischemic brain damage was quantified using the TTC staining technique. As shown in Fig. 4, there was no infarct damage in the sham rats. The cerebral infarct area of rats in the MCAO group was significantly increased compared with that in sham group. By contrast, treatment with luteoloside or the positive control drug nimodipine significantly reduced the cerebral infarct area compared with the MCAO treatment.

3.4. Luteoloside attenuated MCAO-induced neuroinflammation

To determine whether MCAO surgery triggered neuroinflammation, we investigated the production of IL-1β and TNF-α as well as the expression of iNOS and COX-2 in the rat brain after 24 h of reperfusion. The results showed that the levels of IL-1β and TNF-α were significantly increased in brain tissue homogenates of MCAO-treated rats, while luteoloside treatment significantly inhibited IL-1β (Fig. 5A) and TNF-α (Fig. 5B) release compared with MCAO treatment. We also found that the protein expression of iNOS and COX-2 was significantly enhanced in MCAO-treated rats compared with that in the sham group by Western blot. Luteoloside treatment significantly reversed the increases in iNOS and COX-2 expression observed in MCAO-treated rats (Fig. 5C and D).

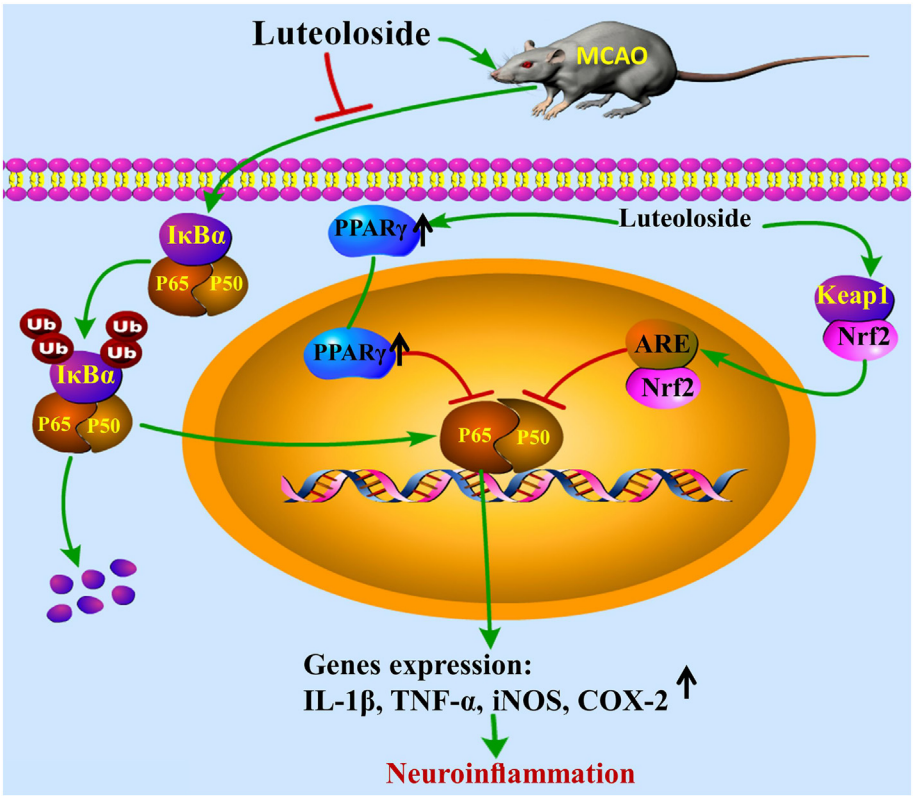

Fig. 9. Possible mechanisms of protective effects of luteoloside on acute focal cerebral ischemic injury induced by MCAO. Luteoloside inhibited the neuroinflammation by upregulating the protein expression of PPARγ, increasing Nrf2 nuclear accumulation and suppressing the NF-κB signaling in MCAO rat.

3.5. Luteoloside suppressed the activation of the NF-κB pathway induced by MCAO

NF-κB signaling is involved in the production of proinflammatory mediators. The activation of NF-κB is initiated by the phosphorylation of IκB and the release of NF-κB, which can translocate into the nucleus. Accordingly, we measured the effect of luteoloside on the NF-κB pathway in rat brains injured by MCAO. As shown in Fig. 6, the phosphorylation of IκBα was markedly increased after reperfusion for 24 h, whereas treatment with luteoloside remarkably alleviated this alteration. Additionally, the nuclear localization of NF-κBp65 was significantly enhanced in MCAO rat brains; however, luteoloside suppressed the phosphorylation of NF-κBp65 (Fig. 6).

3.6. Luteoloside upregulated the protein expression of PPARγ in MCAO-treated rats

Some researchers believe that the neuroprotective effect of PPARγ is mainly related to its regulation of the inflammatory response [26]. Next, we investigated the expression of PPARγ by Western blot assay. At 24 h after reperfusion, the level of PPARγ in the MCAO group was significantly decreased compared with that in the sham group. Luteoloside at doses of 20, 40, and 80 mg/kg significantly increased PPARγ expression in the brains of MCAO-treated rats (Fig. 7).

3.7. Luteoloside prompted the MCAO-induced nuclear translocation of Nrf2

Accumulating evidence suggests that activation of the Nrf2 pathway plays a beneficial role in the inflammatory response; thus, we investigated the effect of luteoloside on the Nrf2 pathway in the brains of MCAO-injured rats. As shown in Fig. 8, the proportion of Nrf2 in the nuclear fraction of the cerebral homogenate was significantly enhanced by luteoloside treatment, suggesting that luteoloside prompts the translocation of Nrf2 from the cytoplasm to the nucleus.

4. Discussion

Cerebral ischemia remains the leading cause of morbidity and mortality worldwide, and no available drugs have been shown to be effective in clinical trials of ischemic stroke [1]. Therefore, the search for promising and safe neuroprotective agents is particularly urgent and important. Recently, many natural herbal extracts have attracted increasing attention because of their outstanding biological activities in many diseases. Luteoloside is a flavonoid compound that is widely found in plants such as honeysuckle, chrysanthemum, and celery and has anti-oxidant, anti-inflammatory and other activities [20,27]. Research in recent years has shown that luteoloside also exerts cardioprotective effects in vitro [20], but there are few studies on its neuroprotective effect. Here, we demonstrated for the first time that luteoloside has neuroprotective effects in rats with cerebral I/R injury. The experimental data revealed that the administration of luteoloside could improve neurological function and decrease brain edema after cerebral I/R injury. TTC and HE staining of brain tissues also confirmed the therapeutic effect of luteoloside on MCAO rats.

Inflammatory responses play crucial roles in pathophysiology following stroke [2]. In response to ischemic stroke, a series of intracellular signaling cascades augment the release of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and the expression of neuroinflammatory genes including COX-2 and iNOS, ultimately leading to inflammatory responses; this, in turn, exacerbates irreversible brain damage [3,28–30]. In this study, our results showed that luteoloside treatment reduced the secretion of TNF-α and IL-1β and inhibited the expression of the inflammatory mediators iNOS and COX-2 in an animal model of acute ischemic stroke. These data indicate that luteoloside could protect nerve function from damage by inhibiting neuroinflammation induced by cerebral ischemia reperfusion.

NF-κB plays an important role in many diseases by regulating inflammatory gene expression. In inactive cells, NF-κB dimers interact with IκBα to form inactive complexes that are localized to the cytoplasm. When the cells are exposed to anoxia, NF-κB is activated via rapid phosphorylation, ubiquitinylation, and ultimately proteolytic degradation of IκBα, which causes the translocation of NF-κB from the cytoplasm to the nucleus to regulate inflammatory cytokine gene transcription and the expression of proinflammatory cytokines [31]. To investigate the anti-inflammatory mechanism of luteoloside, the effects of luteoloside on the NF-κB signaling pathway were measured. Our results showed that luteoloside significantly inhibited the phosphorylation of IκBα and NF-κB p65 in the rat brain after cerebral ischemic injury. The results suggest that NF-κB activation induced by MCAO is significantly inhibited by treatment with luteoloside.

PPARγ belongs to a superfamily of ligand-activated nuclear transcription factors that regulate the transcription and expression of multiple genes and are involved in various physiological, pathological and inflammatory processes. PPARγ is crucial for neuroprotection, antioxidative stress, anti-inflammation, lipogenesis and metabolism [32,33]. Studies have shown that PPARγ activation inhibits NF-kB and JAK-STAT signaling pathways to reduce the release of inflammatory cytokines such as TNF-α, IL-1β and INF-γ, thereby reducing tissue inflammation [34–36]. In this study, we examined PPARγ expression in rats after cerebral I/R injury. As expected, the protein level of PPARγ in the brain of rats undergoing MCAO surgery was downregulated, while luteoloside treatment significantly increased PPARγ expression. This result indicated that the anti-inflammatory effect of luteoloside may be related to the upregulation of PPARγ expression.

Nrf2 is a transcription factor implicated in the transactivation of gene-coding, detoxifying enzymes [37,38]. Nrf2 is sequestered in the cytoplasm by binding to a cytoskeletal protein, Keap1. Once activated, Nrf2 is released from Keap1 and translocates to the nucleus to regulate the expression of Nrf2-responsive genes. Recently, Nrf2 has been reported to be involved in the inflammatory response and can inhibit the activation of NF-κB [39–41]. To further investigate the mechanism of luteoloside neuroprotection, the effect of luteoloside on Nrf2 activation was also measured. The results of our studies showed that Nrf2 expression levels in the nuclear fraction of cerebral homogenate were significantly enhanced by luteoloside treatment, implying that Nrf2 is activated by luteoloside after ischemic injury and that it protects against cerebral I/R injury. However, whether the activation of Nrf2 is involved in the luteoloside-mediated inhibition of MCAO-induced inflammation and Nrf2 interacts with NF-κB require further study.

In conclusion, luteoloside alleviates brain lesions induced by cerebral I/R injury via inhibiting the inflammatory response. The mechanism involves the inhibition of NF-κB pathway activation, increased PPARγ expression and the nuclear translocation of Nrf2 (Fig. 9). Further research will be carried out to assess whether Nrf2 activation is involved in the inhibition of inflammation in cerebral ischemic injury and to determine the therapeutic effect of luteoloside in the prevention of cerebral ischemia-related neurological disorders.

Acknowledgements

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Conflict of interest

The authors declare that they have no competing interests.

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